

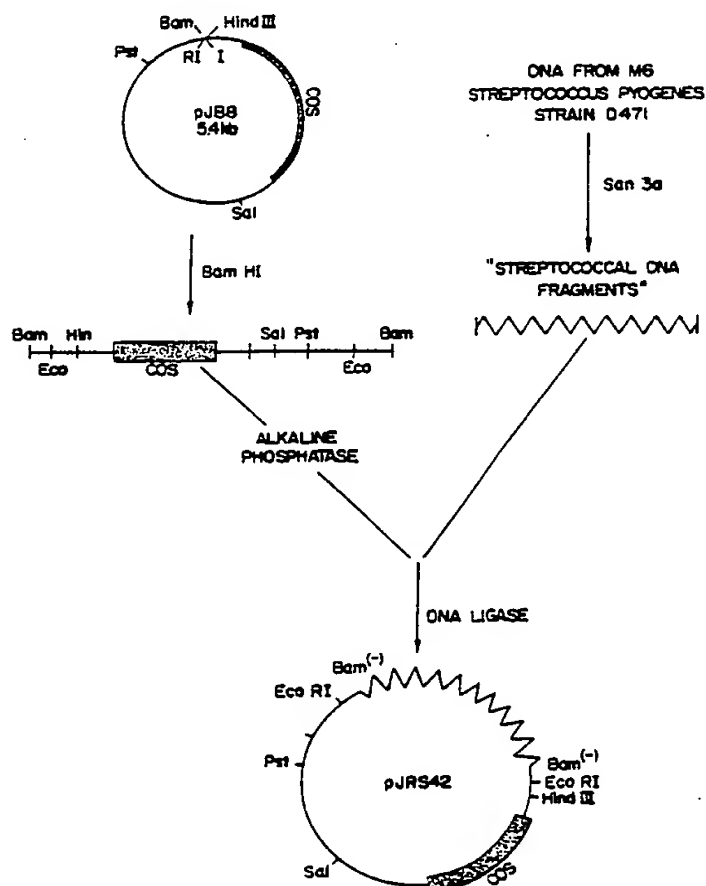


## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification<sup>3</sup> :</b> C12P 21/00, C12N 15/00 C12N 1/20, 1/00 C12Q 1/68, 1/14 C07H 15/12, 17/00 C07G 7/00, A61K 39/02	<b>A1</b>	<b>(11) International Publication Number:</b> WO 85/ 00832  <b>(43) International Publication Date:</b> 28 February 1985 (28.02.85)
<b>(21) International Application Number:</b> PCT/US84/01261 <b>(22) International Filing Date:</b> 9 August 1984 (09.08.84)  <b>(31) Priority Application Numbers:</b> 521,962 621,716 <b>(32) Priority Dates:</b> 10 August 1983 (10.08.83) 18 June 1984 (18.06.84) <b>(33) Priority Country:</b> US  <b>(71) Applicants:</b> THE ROCKEFELLER UNIVERSITY [US/US]; 1230 York Avenue, New York, NY 10021 (US). EMORY UNIVERSITY [US/US]; 1380 South Oxford Road, Atlanta, GA 30322 (US).  <b>(72) Inventors:</b> SCOTT, June, R. ; 1500 Lively Ridge Road, Atlanta, GA 30329 (US). FISCHETTI, Vincent, A. ; 448 Joan Court, West Hempstead, NY 11552 (US).		<b>(74) Agents:</b> SCOBEEY, Robert et al.: Wyatt, Gerber, Shoup, Scobey & Badie, 261 Madison Avenue, New York, NY 10016 (US).  <b>(81) Designated States:</b> AT (European patent), AU, BE (Eu- ropean patent), CH (European patent), DE (Euro- pean patent), DK, FR (European patent), GB (Euro- pean patent), JP, LU (European patent), NL (Euro- pean patent), SE (European patent).  <b>Published</b> <i>With international search report.</i>

**(54) Title:** PRODUCTION OF STREPTOCOCCAL M PROTEIN IMMUNOGENS AND MOLECULAR PROBES**(57) Abstract**

Methods and compositions for the cloning and expression of *Streptococcus pyogenes* M protein genes, and, in particular, types 5, 6 and 24 gene in single-cell host organisms. The streptococcal M protein produced by the recombinant DNA techniques described herein may be formulated for use as immunogens in vaccines to protect against *S. pyogenes* infections. The gene for the M protein may further be employed as a molecular probe for the accurate identification of streptococci in infected body tissues and fluids.



***FOR THE PURPOSES OF INFORMATION ONLY***

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	KR	Republic of Korea
AU	Australia	LI	Liechtenstein
BE	Belgium	LK	Sri Lanka
BG	Bulgaria	LU	Luxembourg
BR	Brazil	MC	Monaco
CF	Central African Republic	MG	Madagascar
CG	Congo	MR	Mauritania
CH	Switzerland	MW	Malawi
CM	Cameroon	NL	Netherlands
DE	Germany, Federal Republic of	NO	Norway
DK	Denmark	RO	Romania
FI	Finland	SD	Sudan
FR	France	SE	Sweden
GA	Gabon	SN	Senegal
GB	United Kingdom	SU	Soviet Union
HU	Hungary	TD	Chad
JP	Japan	TG	Togo
KP	Democratic People's Republic of Korea	US	United States of America

-1-

PRODUCTION OF STREPTOCOCCAL M PROTEIN IMMUNOGENS  
AND MOLECULAR PROBES

5           The invention described herein was made in the course of work supported in part by grants from the National Institutes of Health of the Department of Health and Human Services.

10                           1. FIELD OF THE INVENTION

          This invention relates to compositions and processes for use as diagnostic probes and for the production of vaccines utilizing as an immunogen an  
15 antiphagocytic streptococcal protein, such as M6 protein of Group A streptococcus. The M protein is a fibrillar surface molecule which enables streptococcus to resist phagocytosis by macrophages and polymorphonuclear neutrophils of the infected host organism.

20           The present invention utilizes recombinant DNA techniques to insert a DNA sequence coding for M protein, or a portion thereof, into a DNA plasmid, such as viral DNA, plasmid DNA or cosmid DNA, such that the plasmid is  
25 capable of replicating and directing expression of the M protein gene in a bacterial host or other single cell system. The resulting recombinant DNA molecules are introduced into host cells to enable production of M protein, or a portion or molecular variant thereof. The  
30 protein produced is then isolated, purified and modified

SUBSTITUTE SHEET



-2-

for use as an immunogen in a vaccine against streptococcal infection.

5 This invention further provides a method for the  
detection of Group A, C and G streptococci. Such  
detection is achieved through the use of a specific  
molecular probe based on the entire gene or a segment of  
the gene coding for the M protein of Group A  
streptococcus. A DNA probe useful in a hybridization  
10 screening method is described herein for the examination  
of clinical isolates in cases of suspected streptococcal  
infection.

## 2. BACKGROUND OF THE INVENTION

15

It is well recognized that acute rheumatic fever  
and acute glomerulonephritis are sequelae of Group A  
streptococcal infection. In developing countries of the  
tropics and subtropics, rheumatic heart disease is  
20 currently the most common form of cardiac damage.  
Prevalence rates of this disease as high as 22-23 per  
thousand have been reported in school-age children in  
urban slums of some developing countries of the world. It  
is estimated that in India alone, as many as six million  
25 children may be afflicted. Although the exact mechanisms  
of disease causation are not understood, it is clear that  
rheumatic fever, as well as acute nephritis, follows  
infection with Streptococcus pyogenes (Group A  
streptococcus).

30

The streptococcal M protein is the major  
virulence factor of this bacterium by virtue of the fact  
that it imparts to the organism resistance to phagocytic  
attack. Antigenic variation is the primary mechanism by

35



which the Group A streptococcus is able to evade the host's immune response and thus cause disease in man. Resistance to Group A streptococcal infection is dependent upon the presence of type-specific antibodies to the M protein, a fibrillar molecule found on the surface of the organism. In addition to a number of nontypable strains, about seventy distinct Group A streptococcal M types are currently recognized. Despite the fact that antibodies cross-reactive among certain M types are common, only antibodies prepared against the homologous type are capable of initiating phagocytosis of the organisms (i.e., are opsonic antibodies). Furthermore, not all homologous, or type-specific antibodies are opsonic.

The fact that specific antiserum can be prepared to Group A streptococci has made it possible to detect streptococcal infection by subjecting clinical isolates, such as those obtainable by throat swab, to serological testing. The identification of group A streptococci in an infection requires the isolation of the organism in pure culture, extraction of the group-specific carbohydrate, and reaction with group-specific antiserum. A clinical test for streptococcal infection that could be based upon a property common only to all pathogenic strains would thus be highly desirable.

## 2.1. RECOMBINANT DNA TECHNOLOGY AND GENE EXPRESSION

Recombinant DNA technology involves the technique of DNA cloning whereby a specific DNA fragment is inserted into a genetic element called a vector which is capable of replication and transcription in the host cell. The vector can be either a plasmid or a virus. Plasmids are

small, circular molecules of double-stranded DNA that occur naturally in both bacteria and yeast, where they replicate as independent units as the host cell proliferates. These plasmids generally account for only a small fraction of the total host cell DNA, and often carry genes that confer resistance to antibiotics. These genes, and the relatively small size of the plasmid DNA, are exploited in recombinant DNA technology.

10           The inserted DNA fragment of a recombinant DNA molecule may be derived from an organism which does not exchange information in nature with the host organism, and may be wholly or partially synthetically made. Construction of recombinant DNA molecules using  
15   restriction enzymes and ligation methods to produce recombinant plasmids has been described in U.S. Pat. No. 4,237,224, issued to Cohen and Boyer. The recombinant plasmids thus produced are introduced and replicated in unicellular organisms by means of transformation. Because  
20   of the general applicability of the techniques described therein, U.S. Pat. No. 4,237,224 is hereby incorporated by reference into the present specification.

          A different method for introducing recombinant  
25   DNA molecules into unicellular organisms is described by Collins and Hohn in U.S. Pat. No. 4,304,863 which is also incorporated herein by reference. This method utilizes a packaging/transduction system with bacteriophage vectors.

30           Because it is supercoiled, plasmid DNA can easily be separated from the DNA of the host cell and purified. For use as cloning vectors, such purified plasmid DNA molecules are cut with a restriction nuclease and then

SUBSTITUTE SHEET



-5-

annealed to the DNA fragment that is to be cloned. The hybrid plasmid DNA molecules produced are then reintroduced into bacteria that have been made transiently permeable to macromolecules (competent). Only some of the treated cells will take up a plasmid and these cells can be selected for the antibiotic resistance conferred on them by the plasmid since they alone will grow in the presence of antibiotic. As these bacteria divide, the plasmid also replicates to produce a large number of copies of the original DNA fragment. At the end of the period of proliferation, the hybrid plasmid DNA molecules are purified and the copies of the original DNA fragments are excised by a second treatment with the same endonuclease.

Regardless of the method used for construction, the recombinant DNA molecule must be compatible with the host cell, i.e., capable of autonomous replication in the host cell. The recombinant DNA molecule should also have a marker function which allows the selection of host cells transformed by the recombinant DNA molecule. In addition, if all of the proper replication, transcription and translation signals are correctly arranged on the plasmid, the foreign gene will be properly expressed in the transformed cells and their progeny.

## 2.2. VACCINES

Vaccines are an approach to the control and prevention of diseases. Vaccines can be prepared by mixing the immunogenic portion of an antigen with an adjuvant. This preparation, when injected into a host animal or man, induces the production of antibody to the antigen, and thus provides active immunization to the disease caused by the relevant organism expressing the antigen.

-6-

Peptide vaccines contain only the necessary and relevant immunogenic material, such as portions of the surface proteins of bacteria and viruses. Peptide vaccines can be made by isolating the relevant peptide  
5 from a highly purified bacterial fraction, or by synthesizing the relevant polypeptide. A major advantage of peptide vaccines is the exclusion of unrelated material of bacterial origin and of host- or donor-derived interfering substances. However, at present, production  
10 of peptide vaccines using these methods is generally too expensive for widespread commercial use. Recombinant DNA technology has much to offer in the production of peptide vaccines; the molecular cloning and host cell expression of bacterial genes which encode the relevant immunogenic  
15 portions of the bacteria can produce sufficient quantities of the relevant immunogen for use in a peptide vaccine.

Vaccines are often administered in an emulsion with various adjuvants. The adjuvants aid in attaining a  
20 more durable and higher level of immunity using smaller amounts of antigen in fewer doses than if the immunogen were administered alone. The mechanism of adjuvant action is complex and not completely understood. However, it may involve the stimulation of phagocytosis and other  
25 activities of the reticuloendothelial system as well as a delayed release and degradation of the antigen. Examples of adjuvants include Freund's adjuvant (complete or incomplete), Adjuvant 65 (containing peanut oil, mannide monooleate and aluminum monostearate), and mineral gels  
30 such as aluminum hydroxide, aluminum phosphate, or alum. Freund's adjuvant is no longer used in vaccine formulations for humans or for food animals because it contains nonmetabolizable mineral oil and is a potential carcinogen; however, the mineral gels are widely used in  
35 commercial veterinary vaccines.





### 2.3. ATTEMPTS TO DEVELOP A STREPTOCOCCAL VACCINE USING M PROTEIN ANTIGEN

5 Fox, J. Immunol. 93:826-837 (1964) has used  
M proteins purified from streptococci as immunogens in  
rabbits to induce type-specific bactericidal antibodies.  
However, attempts at vaccinating humans with partially  
purified streptococcal M proteins have been met with  
varied success since strong local and systemic reactions  
usually occur in recipients. See Schmidt, J. Infect. Dis.  
10 106:250-255 (1960) and Potter et al., J. Clin Invest.  
41:301-310 (1962). Fox et al., J. Infect. Dis.  
120:598-604 (1969) and Fox et al., J. Exp. Med.  
124:1135-1151 (1966), using purified acid-extracted  
M protein, were partially successful with their vaccine.  
15 Of 22 adults vaccinated, 15 responded with a secondary  
rise in type-specific antibody titer; however, only 5  
exhibited a rise in bactericidal antibodies.

Beachey et al., J. Exp. Med. 150:862-877 (1979)  
20 vaccinated 12 adults with an alum precipitated preparation  
of a pepsin-derived fragment of the M24 protein (Pep  
M24). This was considered well tolerated since no local  
or systemic reactions were observed. Ten of the 12  
persons vaccinated responded by developing M24  
25 type-specific opsonic antibodies.

Immunological studies by Dale et al., J. Exp.  
Med. 151:1026-1037 (1980) revealed that 2 of the 12  
volunteers, though immunized with M24, also developed  
30 antibodies that bind to both M5 and M6 proteins, of which  
only the M6 was opsonic. However, Beachy et al., in  
Symposium on Bacterial Vaccine, Ed. J.B. Robbins, J.C.  
Hill, Brian Decker Publisher, New York, pages 401-410  
(1981), found that of four rabbits immunized with purified  
35

-8-

Pep M5 protein (pepsin-derived fragment of the M5 protein), one produced antibodies directed against cardiac tissue in high titer. This antisera cross-immunoreacted with type M5 protein and cardiac tissues.

5

#### 2.4. DNA HYBRIDIZATION ASSAYS

A general diagnostic method for the detection of pathogenic microorganisms may be devised if a DNA segment of the genome expected to be found in such organisms is available in pure form. If it is, the DNA segment may be used as a hybridization probe, by tagging it with a chemical, enzymatic or radioisotopic reporter group.

15 Grunstein and Hogness [Proc. Natl. Acad. Sci. U.S.A. 72: 3961 (1975)] have used this approach in a method called colony hybridization, where bacteria to be assayed were transferred to a nitrocellulose filter. The colonies on the filter were then lysed, and the genomic DNA released was fixed to the filter. The presence of nucleotide sequences in the affixed DNA that were complementary to the sequence of a <sup>32</sup>P-labeled probe was then monitored by autoradiography. Other general aspects of DNA hybridization have been described by Falkow et al. in U.S. Patent No. 4,358,535.

#### 3. SUMMARY OF THE INVENTION

Methods and compositions are provided for the cloning and expression of the streptococcal M protein gene in single cell organisms. Also described are methods for culturing these novel single-cell organisms to produce M protein, a rapid assay for identifying single colonies which express the M protein DNA, and a method for identification of the gene product. The M protein



produced by the recombinant DNA techniques described herein may be formulated for use as an immunogen in a vaccine to protect against Streptococcus pyogenes infection.

5

In a particular embodiment disclosed herein, the protein produced by E. coli transductants is slightly larger than the M6 protein isolated by solubilization of the Group A streptococcal cell wall, but similar in size to that secreted by streptococcal protoplasts and L-forms. Immunologically, the molecule synthesized by E. coli transductants has the same type-specific determinants as the streptococcal M6 protein. The M protein was characterized antigenically by Ouchterlony double diffusion experiments and immunogenically by (a) an opsonic antibody removal test and (b) ability to elicit production of opsonic antibodies. The cloned M protein was isolated and then sized by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Additionally, methods are described for isolating the expressed gene product.

The present invention provides a method of producing streptococcal opsonic antibodies and antigens of general importance in human medicine and in microbiological research. This includes use of streptococcal M proteins produced by the present invention as highly reproducible standard antigens for ultrasensitive assays such as radioimmunoassays. These assays may be used as diagnostic tools for detection of antibodies to streptococcus in biological samples.

Through the use of the streptococcal M protein gene or a fragment thereof as a molecular probe, a method is also provided for the diagnostic identification of pathogenic streptococci in body tissues and fluids. In

-10-

this method, DNA is extracted from microbial isolates and examined for complementary nucleotide sequences by hybridization to the molecular probe. By this means large numbers of isolates may be screened for the presence of streptococci with relative ease, high sensitivity and accuracy. The result is a screening test offering marked advantages over the more cumbersome serological diagnostic tests presently in use.

10                   4. BRIEF DESCRIPTION OF THE FIGURES

The present invention may be more fully understood by reference to the following detailed description of the invention and the figures in which:

15                   FIG. 1 (not to scale) represents the construction of pJRS42, a recombinant plasmid derived from streptococcal DNA fragments and pJB8. (See Section 6.2.) Various restriction sites within the streptococcal DNA are not shown.

20

FIG. 2 represents a restriction map of pJRS42.13. This plasmid was derived from pJRS42 by digestion with EcoRI to remove unnecessary streptococcal DNA, then religated. Plasmid pJRS42.13 has only a single EcoRI site. (See Section 6.3.)

25

FIG. 3 is a restriction map of various subclones of plasmid pJRS42.13, which contains the genes coding for the M6 protein of type 6 Group A streptococcus. The clear boxes define clones that express M6 protein, and the shaded boxes denote clones that do not. The vector pBR322 was used in all cases except 42.21 and 42.19, which are in M13 and in pUC8 and pUC9, respectively. The arrow above the restriction map indicates the direction of

30

35

-11-

transcription of the gene encoding type 6 M protein (emm6), and the approximate extent of the gene. The end of the molecule corresponding to the amino terminus of the protein is denoted by "N".

5

FIG. 4 is a representation of a portion of the DNA sequence of the emm6 gene that encodes the amino terminal end of the M6 protein, as determined by the method of Sanger et al. [Proc. Natl. Acad. Sci. U.S.A. 74: 5463 (1977)], and the amino acid sequence of the protein as predicted from the DNA sequence. The amino terminal amino acid, as determined by sequential Edman degradation (reference), is indicated by an "N" below the amino acid sequence.

15

FIG. 5 represents agar gel electrophoretic analysis of DNA hybridization with the NciI/PvuII emm6 DNA probe from the restriction enzyme digestion of plasmid pJRS42.13 of FIG. 3. The DNA in each lane is as follows: lane 1, oligonucleotide size standards of 10.90, 7.74, 5.15, 2.44, 1.80 and 0.60kb; lane 2, M6 strain D471; lane 3, M47; lane 4, M5; lane 5, M19; lane 6, M26; lane 7, M11; lane 8, M24; lane 9, M12; lane 10, M23; lane 11, M28 (from M-strain T28/51/4); and lane 12, M28 (from M+ strain T28/150A/5).

25

## 5. DETAILED DESCRIPTION OF THE INVENTION

### 5.1. M PROTEIN IMMUNOGENS

30

This invention relates to the use of recombinant DNA techniques to produce streptococcal M proteins which can be used as immunogens in vaccine formulations. More specifically, the production of M6 protein is described.

35

-12-

The recombinant plasmids, constructed as described herein, provide for host cell (procaryotic or eucaryotic) production of a streptococcal M protein which is stable and resistant to host cell degradation. Such  
5 plasmids enable the generation of large quantities of protein, or a fragment thereof, containing immunological and antigenic determinants of naturally-occurring M protein. The particular embodiment described herein concerns M6 protein. However, the DNA molecules described  
10 herein are not limited to the production of M6 protein and may be used to produce any of the Group A streptococcal M proteins.

Generic applicability to all M proteins of the  
15 compositions and methods disclosed herein for M6 protein is apparent from the research of Fischetti and Manjula (1982, Biologic and Immunologic Implications of the Structural Relationship Between Streptococcal M Protein and Mammalian Tropomyosin, pp. 411-448 in Robbins, Hill  
20 and Sadoff (eds.), Bacterial Vaccines). For example, all of the M proteins sequenced so far, including M5, M6 and M24, exhibit significant homology and all are coiled-coil structures. The amino terminal and other sequenced segments of the three M molecules reveal that they all  
25 exhibit a repeating seven-residue periodicity. Additionally, immunologic analysis of several M types has revealed cross-reactions among various types. Fischetti, J. Exptl. Med. 146:1108-1123 (1977); Manjula and Fischetti, J. Exptl. Med. 151:695-708 (1980); Manjula and  
30 Fischetti, J. Immunol. 124:261-267 (1980). Furthermore, the hybridization data presented below indicates structural similarity among all 56 M protein genes tested.

It can readily be seen by those skilled in the art that various immunogens and vaccine formulations can be prepared.

5

## 5.2. M PROTEIN MOLECULAR PROBE

The M protein gene described herein, or a fragment thereof, may be used as a molecular probe in a diagnostic test for streptococci. The basis of the test is the fact that pathogenic streptococci possess gene sequences that are complementary to segments of the M protein gene of this invention. Such gene complementarity may be readily detected by isolating microbial DNA from a suspected streptococcal infection and annealing the DNA with the molecular probe under appropriate conditions on a solid support or in liquid medium. The occurrence of hybridization may readily be detected by attachment to the probe of a suitable reporter group.

Streptococcal organisms might be isolated from a point of infection in any body tissue or fluid, but a simple throat swab would be the most likely source. The microorganisms thereby obtained, which would probably be a mixed culture, can be used directly (e.g., on the swab) or grown first to produce more cells in any of the culture media that are well known to those skilled in the art. Then, the DNA from the mixed culture can be extracted, following disruption by freeze-thawing, sonication or other mechanical means and/or by treatment with general or specific agents that lyse bacterial cell walls.

The extracted DNA is generally denatured by the addition of aqueous alkali and then washed with a buffered

-14-

solution. The particular concentrations of alkali, buffer constituents, etc., depend on the conditions of the experiment and may be readily determined by routine experimentation. The denatured M protein gene probe of  
5 this invention may then be added to the microbial DNA preparation and permitted to hybridize at points of DNA sequence complementarity. Specific binding will be recognized as that remaining after extensive washing of the hybridization mixture to eliminate non-specific  
10 binding.

The hybridization may be carried out in one of numerous solutions that have been developed and are acceptable for the purpose. Falkow et al. have described  
15 many of the considerations of solution composition and the hybridization process in general in U.S. Pat. No. 4,358,535, which because of its general utility is hereby incorporated by reference. The specific values of other hybridization parameters, such as time and temperature,  
20 and the method employed are not essential to this invention. Methods described by Gall and Pardue [Proc. Natl. Acad. Sci. U.S.A. 63:378-383 (1969)] and by John et al. [Nature 223:582-587 (1969)], for example, can be applied. Indeed, it is expected that the method of choice  
25 for hybridization will change as the state of that art advances.

The molecular probe upon which the present invention is based may encompass all or only a portion of  
30 the M protein gene, as long as enough of the gene sequence is retained to permit specific hybridization to occur. Such hybridization may be detected even at extremely low levels by coupling an appropriate reporter group to the molecular probe. The probe may be radiolabeled (e.g.

35



-15-

labeled with  $^{32}\text{P}$ ,  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{35}\text{S}$  etc.), or it may be tagged with a chemical or enzymatic reporter group. For example, colorimetric detectors coupled to biotinylated probes may be used in conjunction with avidin derivatives  
5 such as fluorescein-avidin, rhodamine-avidin, or enzyme-linked avidin.

The following Example of a method for the cloning and expression of the M6 protein in Escherichia coli and  
10 for the use of the M6 protein gene as a probe to characterize streptococcal infection is given for purposes of illustration and not by way of limitation on the scope of the invention.

15 6. EXAMPLE: PREPARATION OF THE CLONE CONTAINING THE STREPTOCOCCAL M PROTEIN GENE, AND USE OF THE GENE AS A MOLECULAR PROBE IN A DIAGNOSTIC TEST FOR STREPTOCOCCAL INFECTION

6.1. ISOLATION OF STREPTOCOCCAL DNA

20 The source of the M6 protein gene was Streptococcus pyogenes strain D471 (Group A streptococcus). Group C streptococcal phage lysin, in the presence of 30% raffinose, was used to solubilize the Group A streptococcal cell wall leaving stable protoplasts  
25 (Phillips et al., Proc. Natl. Acad. Sci., U.S.A. 78:4689 (1981)). The protoplasts were then washed extensively and treated with proteinase K to remove streptococcal DNase. Protoplasts were lysed by dilution into sodium dodecyl sulfate (SDS) and the extract was treated with  
30 ribonuclease I to digest RNA. Cesium chloride was added. The preparation was centrifuged at about 100 x g to remove protein and dialyzed overnight. The DNA was precipitated with ethanol. The DNA fragments which were selected for use were well over 100 kilobases (kb) before digestion (as

SUBSTITUTE SHEET



-16-

assayed by agarose gel electrophoresis on a 0.4% agarose gel with P1 phage DNA as a 100 kb standard and one-half P1 molecules as a 50 kb standard).

5

## 6.2. CLONING INTO E. COLI

A large piece of streptococcal DNA was to be cloned in order to reduce the number of candidate E. coli clones that needed to be screened for M protein production and to retain regulatory regions associated with the structural gene for M protein. Therefore, a cosmid vector that accepted DNA insertions of 35-40 kb was necessary. The 5.4 kb vector, pJB8, was chosen as the cloning vehicle. This vector had been constructed from the ampicillin resistant plasmid HomerI and a synthetic BamHI linker in the manner described by Ish-Horowitz and Burke, Nucleic Acids Res. 9(13):2989-2998 (1981).

In the present invention, vector pJB8 was digested with BamHI as generally described by Maniatis et al., supra, pp. 104-106) which cleaves the vector at a unique site to generate "cohesive ends". The cleaved vector was treated with alkaline phosphatase (e.g., as in Maniatis, et al., supra, 133-134) to remove the 5'-phosphates of the linearized vector in order to prevent vector-vector religation and recircularization. To generate random DNA fragments, Sau3a restriction enzyme was used to partially digest the streptococcal DNA (e.g., as in Maniatis, et al., supra, 298) isolated as described in Section 6.1. The fragmented streptococcal DNA was ligated into the BamHI site on the pJB8 vector (see FIG. 1, e.g., as in Maniatis, et al., supra, 298-299).

-17-

The vector with streptococcal DNA inserted was packaged into lambda phage heads in vitro [see Hohn and Collins, Gene 11:291 (1980)]. The phage containing packaged chimeric DNA was used to transduce the E. coli K12 restriction-less strain C600NR(lam<sup>b</sup>dacI857)recA which carries a thermally inducible prophage. Ampicillin resistant colonies were selected at 30°C, transferred to the same selective medium and incubated at 30°C overnight to provide a master plate.

10

The possibility that the M protein gene would be expressed in E. coli was high because most gram positive genes that have been cloned in E. coli have been expressed. However, it was unlikely that the M protein would appear on the surface of E. coli since it would have to be transported through the periplasm and the outer membrane, neither of which exist in streptococcus. For that reason, the E. coli master plate was then shifted to 42°C to induce prophage and lyse the host cells to detect expression of the cloned gene. [Shalka and Shapiro, Gene 1:65 (1976)]

20

### 6.3. RAPID ASSAY OF SINGLE COLONIES FOR EXPRESSION OF M PROTEIN

25

A rapid assay was developed to recognize the clone of E. coli expressing M protein. This technique readily distinguished single colonies expressing M protein.

30

The assay involved transferring the lysed colonies to be tested to nitrocellulose filters, rinsing the filters in bovine serum albumin to reduce non-specific affinity of the filter for proteins, reacting the filter with antiserum to purified LysM6 that had been exhaustively pre-absorbed with E. coli cells, rinsing

35

INSTITUTE SHEET



-18-

appropriately, reacting with  $^{125}\text{I}$ -Staphylococcal protein A (which binds to the antigen-antibody complexes), rinsing again, and scoring by autoradiography.

5           To detect the M6 protein, the antiserum was diluted over 1000-fold. There was no detectable reaction with E. coli when the antiserum was used at a 10-fold dilution (over 100 times more concentrated). By this method, production by an E. coli clone of less than 1% of  
10 the amount of M6 protein produced in the parental streptococcus was detectable.

          Among 335 colonies screened, one reacted strongly with antiserum to purified M6 protein. The chimeric  
15 plasmid present in this strain was named pJRS42. The ability of this E. coli clone to produce the M6 protein was stably maintained on subculture in ampicillin-containing medium.

20           Plasmid pJRS42 was treated with EcoRI endonuclease which led to removal of a segment of streptococcal DNA to form pJRS42.13. This plasmid retained all necessary replication functions and the entire sequence coding for the M6 protein with its  
25 promoter system.

#### 6.4. IDENTIFICATION OF THE GENE PRODUCT

E. coli strain C600NR containing pJB8 and C600NR  
30 transductant containing pJRS42 were grown at 30°C to late log phase in Todd-Hewitt broth (beef heart infusion broth) containing ampicillin. The cells were pelleted and washed twice, and lysed with ethylene diaminetetraacetic acid (EDTA)-lysozyme followed by freezing in dry ice-ethanol

35



-19-

and thawing quickly at 37°C. Following treatment with DNase, the cellular debris was removed by centrifugation at 10,000 x g for 30 minutes and the extract was passed through a 0.45 micron Millipore filter and dialyzed  
5 against 50mM ammonium bicarbonate.

The identification of M protein molecule produced in E. coli was determined by immunoblot analysis. Equivalent protein concentrations, as determined by the  
10 Folin reaction [Lowry et al., J. Biol. Chem. 193:265 (1951)], were applied to a 12% polyacrylamide gel containing SDS. A standard preparation of purified M6 protein extracted from the type 6 streptococcus by solubilization of the cell wall with phage lysin was  
15 applied to an adjacent well as control. After electrophoresis, the separated proteins were transferred to nitrocellulose and unreacted sites on the filter were blocked using Tween 20 (polyoxyethylene sorbitan monolaurate), and the filter was incubated with antiserum  
20 directed against M6 protein extracted by lysin from streptococcus that had been absorbed with E. coli. An enzyme-linked immunoassay using alkaline phosphatase conjugated to goat anti-rabbit IgG was used to detect the bound antibody. Bands were visualized using indoxyl  
25 phosphate as the alkaline phosphatase substrate and nitroblue tetrazolium as the chromophore by the method of Blake et al. [Anal. Biochem. 136:175-179 (1984)]. The M6 antiserum reacted with both the M6 control and the extract of the E. coli clone containing pJRS42, but not with the  
30 extract from the parent E. coli strain containing only the pJB8 vector.

The molecular weight of the M protein produced by the E. coli clone was compared by immunoblot analysis to a

35



-20-

standard preparation of purified LysM6 protein. This molecule is the result of solubilization of the cell wall of the streptococcus with the enzyme phage lysin and purification by column chromatography. It represents the  
5 largest M protein molecule isolated from the streptococcal cell wall.

The M6 protein was purified as follows: The E. coli containing plasmid pJRS42.13 was treated with  
10 lysozyme in the presence of EDTA and 20% sucrose. This allowed the periplasmic contents to be released into the surrounding fluid. Centrifugation of the organisms left the periplasmic contents in the supernatant along with other E. coli associated proteins. Using this technique  
15 it was found that the M protein was in high concentration in the periplasmic space and virtually absent from the cytoplasm. Thus, this method was used to generate the starting material for the M protein purification.

20 The M protein in the crude periplasmic contents was purified from other contaminating proteins by column chromatography. The crude periplasmic preparation was dialyzed against 5mM ammonium bicarbonate buffer pH 5.5 and applied to a column of carboxymethyl cellulose. The  
25 column was washed with three volumes of the same buffer and the adherent proteins eluted in one step with 100mM sodium phosphate pH 7.0. The eluted protein (containing the M protein) was applied directly to a hydroxylapatite column equilibrated in 25mM sodium phosphate pH 7.0. The  
30 column was washed with 2 column volumes of 200mM sodium phosphate pH 7.0 and the adherent M protein was then eluted with 400mM sodium phosphate pH 7.0. This method resulted in a highly purified M protein preparation as determined by SDS-polyacrylamide gel electrophoresis and  
35 sequence analysis.

SUBSTITUTE SHEET



-21-

Amino terminal sequence analysis of the purified coli-synthesized M6 protein yielded a single phenylthiohydantoin (PTH)-amino acid at each degradation step, verifying the homogeneity of the final preparation.

5 In addition, the amino terminal sequence was found to be identical to that of the LysM6 molecule through the first 35 residues sequenced, with the exception of the amino terminal residue. The E. coli molecule has an additional arginine residue at the amino terminus which may be

10 clipped off during purification of the lysine molecule.

The purified LysM6 preparation exhibited a multiple banding pattern previously observed with the M6 molecule, which is probably due to degradation during

15 extraction and purification. The three major bands corresponded to apparent molecular weights of 51,000, 52,000 and 53,000 daltons. The size heterogeneity of the M6 preparation probably resulted from differences at the carboxy-terminal region of the protein since, during amino

20 terminal sequence analysis of this preparation by sequential degradation, only a single amino acid residue was released at each step. Since the bands from the pJRS42-containing clone that reacted with anti-M6 antibodies are all larger (molecular weights 55,000,

25 57,000, 59,000 daltons) than any from the streptococcal preparation, this suggested that pJRS42 contained the entire structural gene for the M6 protein. This was supported by the fact that this molecular size correlated well with the reported size of M protein secreted from

30 protoplasts and L forms of type 12 streptococci (molecular weight 58,000 daltons). Thus, the proteins in the E. coli preparation may be closer to the size of the intact native M molecule than those released by lysis-extraction of streptococcus.

35

SUBSTITUTE SHEET



-22-

In addition, the M protein isolated from secreting streptococcal L forms and protoplasts appeared more homogeneous. There are several possible explanations for the differences in mobilities of the E. coli protein(s) reactive with anti-M6 antibodies from those of the bands of the purified standard M protein. For example: 1) The protein(s) may include a leader sequence that has not been removed in the E. coli but is normally removed in streptococci; 2) the LysM6 molecule may represent a cleavage product produced during attachment to the streptococcal cell wall; 3) the LysM6 molecule may be a partial degradation product produced during purification of the protein; 4) the E. coli protein(s) may be a "fusion" product produced from a promoter in the vector; 5) a weak translational start sequence that is unable to function in streptococcus may be active in E. coli; and/or 6) the "stop" condon normally functional in streptococcus may be suppressed by the supE mutation in the E. coli strain.

#### 6.5. IMMUNOGENIC CHARACTERIZATION OF THE GENE PRODUCT

An Ochterlony immunodiffusion comparison of M protein of E. coli with that extracted by lysin from streptococcus was performed. Well 1 contained unabsorbed rabbit antiserum prepared against lysin-extracted M6 protein synthesized by the streptococcus; Well 2 contained purified lysin-extracted M6 protein; Well 3 contained M6 protein from the E. coli strain C600NR (pJRS42) which had been partially purified by chromatography on DEAE and CM cellulose. The reaction was performed in 1% agar gel prepared at pH 8.6 in 50mM barbitol buffer. The gel was dried and stained with Coomassie blue.



The results of this double diffusion experiments (gel not shown) supported the conclusion that the extract of the E. coli carrying pJRS42 (but not that of the plasmid without insert) contained a molecule with  
5 antigenic determinants common to at least some of those of streptococcal M6 protein. Thus, the M6 protein synthesized in E. coli has at least some of the same type-specific determinants as the M protein extracted from type 6 streptococci, although the E. coli product has a  
10 higher apparent molecular weight.

#### 6.6. BACTERICIDAL EFFECT OF CLONED M PROTEIN

In order to determine if the M protein produced  
15 by the E. coli contained the antigenic determinants necessary to remove opsonic antibodies from both rabbit and human opsonic antiserum, the following absorption experiment was performed.

20 Purified E. coli-synthesized M6 protein was lyophilized in two 30 µg aliquots. Rabbit type 6 opsonic antiserum (0.5 ml) was added to one, and a similar amount of human serum opsonic for type 6 streptococci was added to the other dried protein sample to form a solution. The  
25 tubes were incubated at 37°C for one hour and allowed to remain at 4°C overnight. The precipitate that formed was centrifuged at 20,000 x g and the resulting supernatant was used in a bactericidal assay using type 6 streptococci.

30 The indirect bactericidal assay was carried out as described originally by Lancefield, J. Exptl. Med. 110:271 (1959). Heparinized whole human blood from normal donors was used as a source of phagocytes. Dilutions of type 6 streptococci (100 µl) were mixed with 400 µl of the

SUBSTITUTE SHEET



-24-

human blood in the presence or absence of either absorbed or unabsorbed serum (100  $\mu$ l). The mixture was rotated end over end at 37°C for 3 hr. The surviving organisms were determined by pour plate method. Rotated controls without  
5 antiserum were run to test the ability of the streptococci to grow in the donor's blood.

The M protein produced by E. coli transductants removed the opsonic antibodies from both the rabbit and  
10 human sera. See Table 1. Thus, the anti-phagocytic determinants of the E. coli M protein function similarly to those of the native M6 molecule.

TABLE 1

REMOVAL OF HUMAN AND RABBIT  
OPSONIC ANTIBODIES WITH  
E. COLI PRODUCED M6 PROTEIN

20	<u>Treatment</u>	<u>Colonies Found*</u>	
		<u>Rabbit anti-M6</u>	<u>Human anti-M6</u>
	Inoculum	20	18
	No serum (control)	790	930
	Unabsorbed serum	8	0
	Absorbed with <u>E. coli</u> M6	1800	2890

25

\* Numbers represent colony forming units as assayed by pour plate method after the 3 hour rotation.

30

#### 6.7. PRODUCTION OF TYPE 6 OPSONIC ANTIBODIES

Production of type 6 opsonic antibodies in rabbits after immunization with purified E. coli-produced M6 protein was accomplished as follows. Antisera to the

35

-25-

purified E. coli-produced M6 protein was prepared in New Zealand white rabbits. The primary inoculation consisted of 100 µg of M6 protein emulsified with complete Freund's adjuvant and given subcutaneously at multiple sites. The  
5 animals were boosted after 4 weeks with the same dose of the M6 protein in incomplete Freund's adjuvant. Animals were bled 10 days later.

As determined by bactericidal assay (described  
10 above), rabbits immunized with the E. coli M6 protein developed antibodies which allowed phagocytosis of type 6 streptococci. See Table 2.

15

TABLE 2

PRODUCTION OF OPSONIC ANTIBODIES IN RABBITS  
IMMUNIZED WITH COLI M6 PROTEIN

20

	<u>Colonies</u> <sup>*</sup>
Inoculum	43
No serum (control)	1112
25 Immune serum	0

\*  
30 Protocol is that described in Section 6.6., supra.  
Numbers represent colony forming units as assayed by  
pour plate method after the 3 hour rotation.

## 6.8. DIAGNOSTIC TEST FOR STREPTOCOCCI

### 6.8.1. PREPARATION AND PURIFICATION OF AN M-GENE DNA PROBE

5           To locate the gene encoding type M6 protein  
(emm6), plasmid pJRS42.13, described in Section 6.3, supra,  
was subjected to digestion with various restriction  
enzymes or combinations of them. The DNA fragments thus  
obtained were sized by electrophoresis through 0.8%  
10 agarose gel as described by Maniatis, et al., supra,  
150-161, and then ligated into different vectors. These  
recombinant vectors were transformed into an E. coli K12  
bacterium that was lysogenic for a thermally inducible  
lambda prophage and screened for the production of protein  
15 reactive with anti-M6 protein antiserum, as described in  
Section 6.4.

          The results of M6 protein expression analysis for  
many of these cloned DNA fragments are shown in FIG. 3.  
20 The clones delineated by clear blocks produced a protein  
that was reactive with anti-M6 antiserum, while those  
shown as shaded blocks did not. Although some of the  
reactive clones such as pJRS42.19 contained too little  
streptococcal DNA to encode the entire M6 protein, their  
25 expression products were evidently of sufficient size to  
show antigenic reactivity with the polyclonal antiserum.

          To learn more about the orientation of the emm6  
gene within the cloned DNA fragments, the streptococcal  
30 DNA in plasmid pJRS42.19 was ligated into the BamHI sites  
of plasmids pUC9 and pUC8 [Messing and Vieira, Gene  
19:269-276 (1982)]. The relationship of these plasmids to  
each other is such that inserted DNA is arranged in

-27-

opposite orientations in the plasmids. Anti-M6 antiserum reacts with the products of both of these clones, indicating that the M6 protein fragment is synthesized when the streptococcal DNA is present in either  
5 orientation in these vectors. It thus appears that the inserted streptococcal DNA carries its own promoter. If this conclusion is correct, pJRS42.19 should encode the N-terminus of the M6 protein.

10           Following ligation of the streptococcal DNA fragment of pJRS42.19 into M13mp8 and mp9 [Messing and Vieira, supra], the sequence of the inserted DNA was determined by the Sanger dideoxy method [Sanger et al., Proc. Natl. Acad. Sci. U.S.A. 74: 5463 (1977)]. The  
15 portion of the sequence encoding the amino terminal end of the M6 protein, together with the amino acid sequence specified by it, is shown in FIG. 4. The amino terminal amino acid, as determined by sequential Edman degradation (reference), is indicated by an "N" below the amino acid  
20 sequence. The amino acid sequence determined in this way was identical to that established for the amino terminus of M6 protein that had been extracted either by pepsin treatment or by phage lysis from streptococcal strain D471 [reference]. It was also identical to the amino terminal  
25 region of the M protein produced by recombinant DNA methodology in E. coli in Section 6.4, supra.

          These results showed that the emm6 gene begins within the DNA fragment contained in pJRS42.19.  
30 Comparison of the DNA sequence with the amino acid sequence shown in FIG. 4 further demonstrates that the N-terminus of M6 protein is at a point that is 32 bases to the left of the Nci I site, as shown in FIG. 3.

SUBSTITUTE SHEET



-28-

5 The M6 protein produced in E. coli was shown by sodium dodecyl sulfate polyacrylamide gel electrophoresis [Fischetti et al., J. Exptl. Med. 159:1083-1095 (1984)] to have an apparent molecular weight of 59,000 daltons. This fact would place the other end of the gene sequence at or near the PvuII site in FIG. 3. Further sequence analysis revealed that the nonsense codon terminating the protein, TAA, is located 38 bases to the right of the PvuII site (Hollingshead et al., manuscript in preparation).

10

A suitable probe containing a large portion of the emm6 gene was prepared by treating plasmid pJRS42.13 with NciI and PvuII. The location of this probe within the pJRS42.13 restriction map is indicated by the heavy arrow in FIG. 3. This probe fragment was purified by electrophoresis in 0.8% agarose gel, electroeluted, and passed through an Elutip-d column (Schleicher and Schuell).

15

#### 20 6.8.2. ISOLATION OF BACTERIAL DNA

20

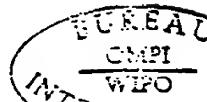
Phage lysin was used to lyse cells of Group A streptococci, by the method of Fischetti et al. [J. Exptl. Med. 133:1105 (1971)]. For other streptococci, overnight Todd-Hewitt-yeast broth cultures (beef heart infusion broth with yeast extract) were diluted ten-fold and grown at 37°C to a cell concentration of about  $5 \times 10^6$  cells per ml. Glycine was added to a concentration of 3% (w/v), and the cells were incubated for 2 additional hours at 37°C. The cells were then washed, sonicated twice for 15-second pulses, and resuspended in 10mM Tris-HCl buffer (pH 8.0) containing 30% (w/v) sucrose and 10 mg/ml lysozyme.

30

After incubation for 30 minutes at 37°C, ethylenediaminetetraacetic acid was added to a final

35

SUBSTITUTE SHEET



concentration of 10mM, and incubation was continued for an additional 30 minutes at 37°C. Protease K (0.1 mg/ml, source) and sodium dodecyl sulfate (1% w/v) were then added, the components were mixed by gentle inversion, and  
5 incubation was continued for another 30 minutes at 37°C. Following this procedure the cell suspensions, which displayed no turbidity, were extracted with phenol:chloroform (10:1) until no protein was visible at the interface. The extracted DNA was then precipitated  
10 with ethanol.

The bacterial strains examined include Staphylococcus aureus, B. subtilis (strain CU1065), Streptococcus pneumoniae and the following streptococcal  
15 strains from The Rockefeller University collection that are standard typing and grouping strains, respectively, used to prepare specific antisera: M1, T1/195/2; M3, B930/61/5; M3R, D58X; M4, T4/95/RB5; M5, T5B/126/4; M6, S43/192/3; M8, C256/86/3; M11, T11/137/3; M12, T12/126/4  
20 (COL 6); M14, T14/46/8; M15, T15/23/7; M18, J17C/55/4; M22, T22/146/1; M23, T23/102/RB5; M24, C98/135/2; M25, B346/136/1; M27, T27/87/1; M28, T28/150A/5; M29, D23; M30, D24/126/3; M31, J137/69/3; M32, C121/39/8; M33, C107/102/2; M36, C119/83/2; M37, C242; M38, C94/80/2; M39, C95/95/1; M40, C143/25/9; M41, C101/103/4; M42, C113/55/5;  
25 M43, C126/170/2; M46, C105/41/5; M47, C744/RB4/6/5; M48, B403/48/5; M49, B737/137/2; M50, B514/33/6; M51, A309/77/1; M52, A871/106/2; M53, A952/94/3; M54, A953/87/3; M55, A928/73/1; M56, A963; M57, A995/91/2; M58, D315/87/3; M60, D335/38/3; M63, D459/50/2; M66, D794/76/2;  
30 M67, D795/95/1; group A, J17A4; group B, 090R; group C, C74; group D, D76; group E, K131; group F, F68C; group G, D166B; group H, F90A; group L, D167A; group M, D168A"x"; group N, C559; group O, B361. The following group A M

-30-

typing strains from the Center for Disease Control, Atlanta, Georgia, were also used: M2, SS633; M9, SS754; M13, SS936; M17, SS631; M19, SS400; M34, SS134; M59, SS913 and M62, SS984.

5

### 6.8.3. DOT BLOT HYBRIDIZATION STUDIES

Dot hybridization was performed on the extracted DNA samples using the procedure for detection of specific DNA sequences described by Kafatos et al. [Nucleic Acid Res. 7; 1541-1552 (1979)]. For a probe, the NciI/PvuII DNA fragment containing most of the emm6 gene (Section 6.8.1) was used following labeling with  $^{32}\text{P}$  by nick translation by the method of Botchan et al. [Cell 9; 269-287 (1976)].

15

To carry out the hybridization, the DNA extracts from the various microbial sources were denatured in 0.6 N NaOH for 15 minutes at room temperature, and then for 10 minutes at 0°C. Then, the samples were neutralized with 2M ammonium acetate, and aliquots of the DNAs were spotted on Biodyne A 0.2 micron nylon filters (Pall Filtration Corp., Glen Cove, N.Y.) in a Bethesda Research Laboratories manifold. Hybridization was carried out by adding at least  $2 \times 10^6$  cpm/filter of the nick-translated  $^{32}\text{P}$  probe in buffer containing 1.8M Tris-HCl with 0.2M Tris base, and the filters were maintained at 64°C overnight. The filters were then washed 10 times with the same buffer at 64°C, dried, and subjected to autoradiography on Kodak XAR-5 film with an intensifying screen. The exposure was carried out at -80°C for 2-4 days.

20

25

30

A summary of the results of these studies is shown in Table 3.

35

SUBSTITUTE SHEET





TABLE 3

## DNA HYBRIDIZATION WITH THE emm6 PROBE

5	Bacterial Type	Strains Showing Hybridization	Strains Showing No Hybridization
10	Group A	1, 2, 3, 4, 5, 6, 8, 9, 11, 12, 13, 14, 15, 17, 18, 19, 22, 23, 24, 25, 27, 28, 29, 30, 31, 32, 33, 34, 36, 37, 38, 39, 40, 41, 42, 43, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 62, 63, 66, 67, 68, A486, A712, D366, D780	
15	M <sup>-</sup> Group A Strains	J17A4, *A486 Var.	T28/51/4
	Other Streptococcal Groups	C, G	B, D, E, F, H, L, M, N, O
20	Other Gram Positive Organisms	None	<u>Streptococcus pneumoniae</u> <u>Staphylococcus aureus</u> <u>Bacillus subtilis</u>
25			

\* Strain A486 Var. is a group A variant strain.

Overall, the dot blot test showed hybridization between the emm6 probe and DNA from 56/56 different M types of group A streptococcus, and from 4 non-typable group A strains and from 2 strains previously characterized as M<sup>-</sup>. No hybridization was seen with DNA from the gram positive organisms Staphylococcus aureus or Bacillus subtilis, from streptococcal Lancefield groups B, D, E, F, H, L, M, N or O, or from Streptococcus

pneumoniae. Hybridization was observed, however, with groups C and G streptococcal DNA. This finding was not unexpected since group C streptococci have occasionally been implicated in human infections, and some strains appear to have a molecule on their outer surfaces which is functionally similar to M protein [Woolcock, Infect. Immun. 10:568 (1974)].

Group G streptococci have also been reported to cause a wide range of human infections. Although it is uncertain whether the virulence of these organisms is always due to the presence of an M-like cell surface protein, it may be that it is. Examination of three strains of group G streptococci isolated from human infections revealed the presence in the strains of a type 12 M cell surface protein [Maxted and Potter, J. Genl. Microbiol. 49:119 (1967)].

Among the group A strains tested were three that are functionally M<sup>-</sup> (i.e., they do not produce protective M protein and are thus phagocytized). The DNA from two of these M<sup>-</sup> strains nevertheless hybridized with the emm6 gene probe, showing that they retain at least some of the emm gene intact. Presumably, these strains are mutants whose emm gene product is either nonfunctional or synthesized in reduced amount. The DNA from one M<sup>-</sup> strain did not hybridize with the probe, suggesting that in that strain the emm gene was substantially deleted.

The results of the dot blot tests were confirmed by a study in which DNA from various M type group A streptococcal strains was extracted and digested with NciI and HindIII. Samples of the resulting fragments of DNA were then separated by agarose gel electrophoresis as described by Maniatis et al., supra, 150-161, and

hybridized with the emm6 <sup>32</sup>P-labeled probe, and the positions of the hybrid DNA fragments were revealed by autoradiography. The results are shown in FIG. 5.

5 In FIG. 5, lane 1 represents 10.9, 7.74, 5.15, 2.44, 1.80 and 0.60 kb DNA molecular size markers that had been labeled with <sup>32</sup>P. Lanes 2 through 10 contained the probe-hybridized NciI/HindIII fragments of DNA from streptococci of M types 6, 47, 5, 19, 26, 11, 24, 12 and 10 23, respectively. In each case, there were two or more DNA fragments that hybridized with the probe. As expected from the dot blot studies, DNA from M<sup>-</sup> strain T28/51/4 (lane 11) did not hybridize, while that from M<sup>+</sup> strain T28/150A/5 (lane 12) did.

15

#### 7. DEPOSITS OF MICROORGANISMS

The following listed E. coli strains carrying the listed plasmid have been deposited with the Agricultural Research Culture Collection (NRRL), Peoria, Ill., and have 20 been assigned the following accession numbers:

<u>E. coli strain</u>	<u>Plasmid</u>	<u>Accession Number</u>
K-12, C600NR Lambda cI857	pJRS42.13	NRRL B-15529
25 K-12, C600NR Lambda cI857	pJRS42	NRRL B-15535

30 The present invention is not to be limited in scope by the microorganisms deposited, since the deposited microorganisms are intended to be illustrative of several aspects of the invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art

35

SUBSTITUTE SHEET



-34-

from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

- 5            In the present specification the base pair sizes given for nucleotides are approximate and are used for purposes of description.

**SUBSTITUTE SHEET**

## WE CLAIM:

1. A process for producing a polypeptide having an immunoreactive and antigenic determinant of a streptococcus M or M-like protein, comprising:
  - (a) culturing a unicellular organism containing a recombinant plasmid comprising a DNA sequence coding for a polypeptide having an immunoreactive and antigenic determinant of a streptococcus protein and capable of being replicated, transcribed and translated in the unicellular organism; and
  - (b) isolating said polypeptide from the culture.
2. The process according to claim 1, wherein said polypeptide is immunogenic.
3. The process according to claim 1, wherein said polypeptide is capable of eliciting opsonic antibodies when used as an antigen.
4. The process according to claim 1, wherein the recombinant plasmid is introduced into the unicellular organism by transformation.
5. The process according to claim 1, wherein the recombinant plasmid is introduced into the unicellular organism by transduction.
6. The process according to claim 1, wherein the recombinant plasmid is introduced into the unicellular organism by transfection.
7. The process according to claim 1, wherein the M protein is from a member of the group A streptococci.



-36-

8. The process according to claim 1, wherein the M-like protein is from a member of the group C streptococci.

5 9. The process according to claim 1, wherein the M-like protein is from a member of the group G streptococci.

10 10. The process according to claim 1, wherein said unicellular organism is a eucaryotic organism.

11. The process according to claim 1, wherein said unicellular organism is a procaryotic organism.

15 12. The process according to claim 11, wherein said procaryotic organism is Escherichia coli.

20 13. A process for preparing a unicellular organism having a DNA sequence coding for a polypeptide having an immunoreactive and antigenic determinant of a streptococcus M protein, comprising: introducing a recombinant plasmid comprising a DNA sequence coding for a polypeptide having an immunoreactive and antigenic determinant of a streptococcus M protein and capable of being replicated, transcribed and translated in the  
25 unicellular organism.

30 14. A purified DNA sequence coding for a Streptococcus pyogenes M protein, or any portion thereof coding for a polypeptide having an immunoreactive and antigenic determinant of a Streptococcus pyogenes M protein.

35 15. The purified DNA sequence according to claim 14, wherein the M protein is from a member of the group A streptococci.

16. The purified DNA sequence according to claim 14, wherein the M-like protein is from a member of the group C streptococci.

5 17. The purified DNA sequence according to claim 14, wherein the M-like protein is from a member of the group G streptococci.

10 18. A recombinant plasmid comprising the DNA sequence of claim 14.

19. A recombinant plasmid of claim 18, further comprising the essential portions of the pJRS42 replicon.

15 20. A recombinant plasmid of claim 18, wherein said DNA sequence is under the control of expression control elements.

20 21. A recombinant plasmid of claim 18, wherein the vector is pJRS42.

22. A recombinant plasmid of claim 18, wherein the vector is pJRS42.13.

25 23. A unicellular organism containing the recombinant plasmid of claim 18.

24. A unicellular organism containing the recombinant plasmid of claim 19.

30 25. A unicellular organism containing the recombinant plasmid of claim 20.

35 26. A unicellular organism containing the recombinant plasmid of claim 21.

SUBSTITUTE SHEET



27. A unicellular organism containing the recombinant plasmid of claim 22.

5 28. An Escherichia coli bacterium containing the DNA sequence of claim 14.

29. An Escherichia coli bacterium containing the recombinant vector of claim 18.

10 30. An Escherichia coli bacterium according to claim 26, deposited with the NRRL and assigned accession No. B-15535, or a mutant, recombinant, or in vivo or in vitro genetically engineered derivative thereof.

15 31. An Escherichia coli bacterium according to claim 27, deposited with the NRRL and assigned accession No. B-15529, or a mutant, recombinant, or in vivo or in vitro genetically engineered derivative thereof.

20 32. A polypeptide having an immunoreactive and antigenic determinant of a Streptococcus pyogenes M protein produced by a unicellular organism of bacterium of claim 23.

25 33. A polypeptide having an immunoreactive and antigenic determinant of a Streptococcus pyogenes M protein produced by Escherichia coli bacterium of claim 29.

30 34. A vaccine formulation comprising the polypeptide of claim 32 and a compatible pharmaceutical carrier therefor.



35. A method for the detection of pathogenic streptococci in a clinical sample suspected of containing said pathogenic streptococci, comprising:

5 (a) contacting an M protein gene or gene fragment probe with a lysate of said sample under conditions which permit hybridization of complementary sequences; and

10 (b) detecting any hybridization which has occurred between said probe and any complementary nucleotide sequence in such clinical sample lysate.

15 36. The method according to claim 35, in which the hybridization is in liquid media.

37. The method according to claim 35, in which the hybridization is on a solid support.

20 38. The method according to claim 35, in which said probe is radiolabeled.

25 39. The method according to claim 35, in which said probe is biotinylated and said hybridization is detected using avidin bound to a colorimetric indicator.

30 40. The method according to claim 35, in which said probe is biotinylated and said hybridization is detected using avidin bound to a fluorescent molecule.

41. The method according to claim 35, in which said probe is the gene encoding M6 protein.

35 42. The method according to claim 35, in which the pathogenic streptococcus detected is group A.



43. The method according to claim 35, in which the pathogenic streptococcus detected is group C.

44. The method according to claim 35, in which  
5 the pathogenic streptococcus detected is group G.

45. The method according to claim 35, in which said sample is obtained by taking a throat swab.

10 46. A purified DNA probe capable of binding to a Streptococcus pyogenes gene coding for M protein.

47. The purified DNA probe according to claim 46, wherein said probe is from an Escherichia coli  
15 bacterium deposited with the NRRL and assigned accession No. B-15529, or a mutant, recombinant, or in vivo or in vitro genetically engineered derivative thereof.

48. The purified DNA probe according to claim 20 46, wherein said probe is from an Escherichia coli bacterium deposited with the NRRL and assigned accession No. B-15535, or a mutant, recombinant, or in vivo or in vitro genetically engineered derivative thereof.

25 49. The purified DNA probe according to claim 46, wherein said probe comprises the DNA sequence of the NciI/Pvu II digestion product of plasmid pJRS42.13.

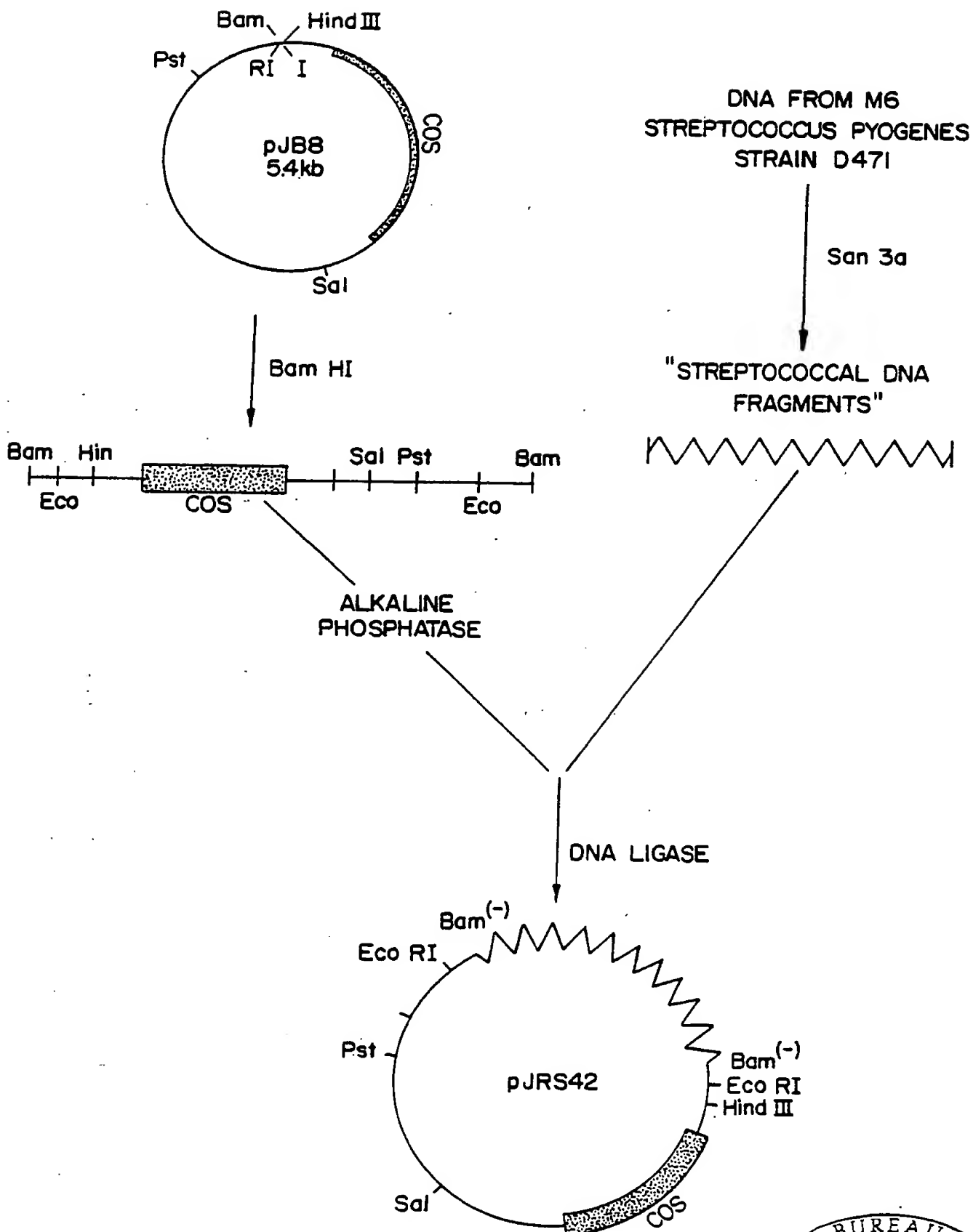
50. The purified DNA probe according to claim 30 46, wherein said probe is coupled to avidin bound to a colorimetric indicator.

51. The purified DNA probe according to claim 35 46, wherein said probe is coupled to avidin bound to a fluorescent molecule.

52. The purified DNA probe according to claim 46, wherein said probe is radiolabeled.

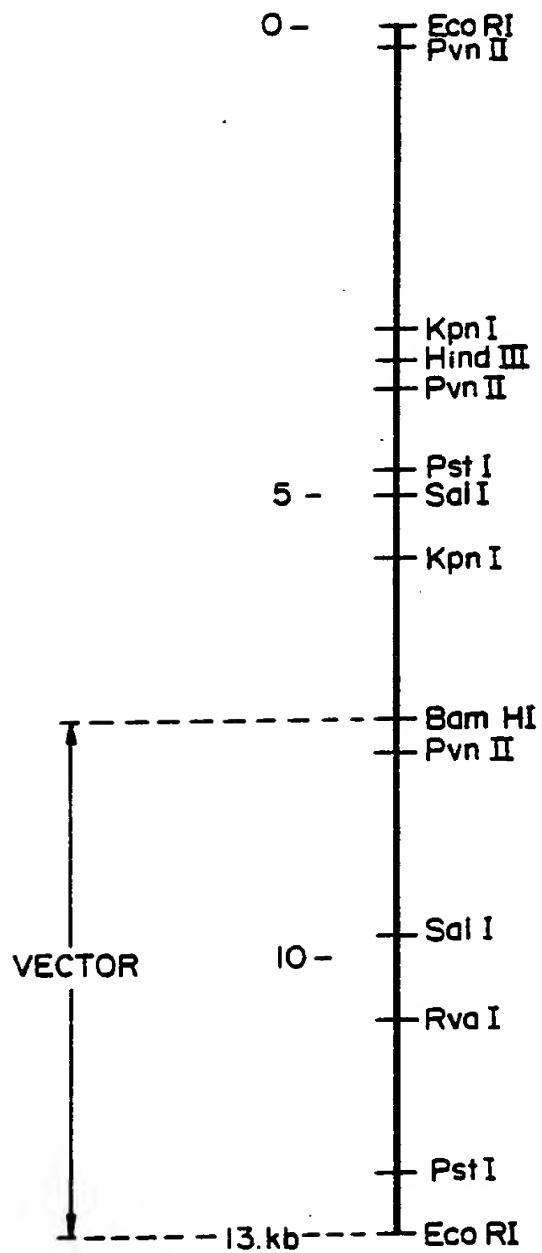
1/4

FIG. 1.



2/4

FIG. 2.



3/4

FIG. 3.

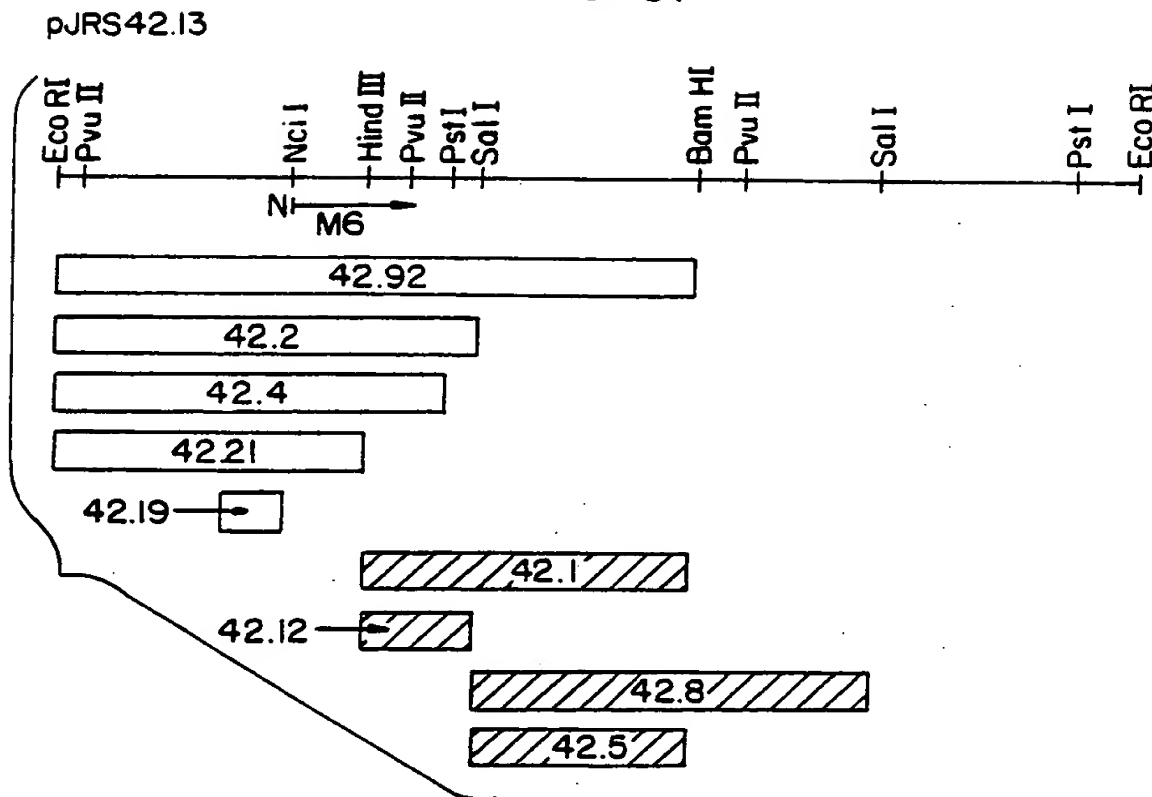


FIG. 4.

172 ATG BCT AAA AAT AAC ACS AAT AGA CAC TAT TCG CTT AGA AAA 213  
Met Ala Lys Asn Asn Thr Asn Arg His Tyr Ser Leu Arg Lys

214 TTA AAA AAA BGT ACT GCA TCA GTA GCA GTG GCT TTG AGT GTA 255  
Leu Lys Lys Gly Thr Ala Ser Val Ala Val Ala Leu Ser Val

256 ATA GGG BCA BGA TTA GTT GTC AAT ACT AAT GAA GTT AGT GCA 297  
Ile Gly Ala Gly Leu Val Val Asn Thr Asn Glu Val Ser Ala

298 AGA GTG TTT CCT AGG GGG ACG GTA GAA AAC CCG GAC AAA GCA 339  
Arg Val Phe Pro Arg Gly Thr Val Glu Asn Pro Asp Lys Ala

N

340 CGA GAA CTT CTT AAC AAG TAT BAC GTA GAG AAC TCT ATG TTA 381  
Arg Glu Leu Leu Asn Lys Tyr Asp Val Glu Asn Ser Met Leu

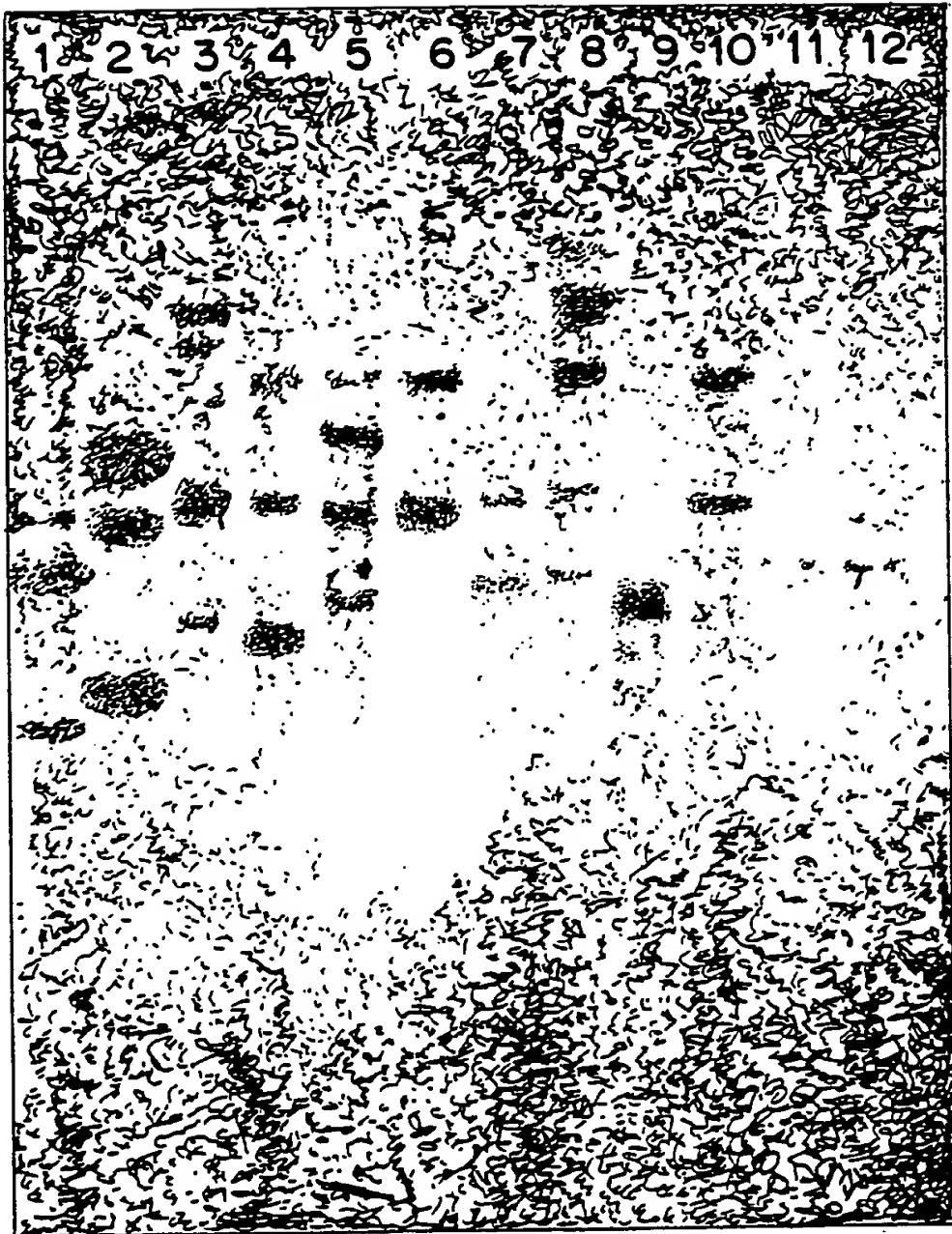
382 CAA GCT AAT AAT GAC AAG TTA ACA ACT GAG AAT AAT AAC TTA 423  
Gln Ala Asn Asn Asp Lys Leu Thr Thr Glu Asn Asn Asn Leu

424 ACA GAT CAG AAT AAA AAC TTA ACA ACT GAG AAT AAA AAC TTA 465  
Thr Asp Gln Asn Lys Asn Leu Thr Thr Glu Asn Lys Asn Leu

466 ACA GAT C 472  
Thr Asp

4/4

FIG. 5.



SUBSTITUTE SHEET

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US84/01261

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>3</sup> According to International Patent Classification (IPC) or, to both National Classification and IPC Int. Cl. <sup>3</sup> C12P 21/00; C12N 5/00; 1/20, 1/00 C12Q 1/68, 1/14 C07H 15/12, 17/00; C07G 7/00; A61K 39/02																																
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Minimum Documentation Searched <sup>4</sup></div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 25%; border-bottom: 1px solid black;">Classification System</th> <th style="border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="border-right: 1px solid black; padding: 5px;">U. S.</td> <td style="padding: 5px;">435/68, 172.3, 253, 317, 6, 36 536/27; 260/112R; 424/92</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>5</sup></div>			Classification System	Classification Symbols	U. S.	435/68, 172.3, 253, 317, 6, 36 536/27; 260/112R; 424/92																										
Classification System	Classification Symbols																															
U. S.	435/68, 172.3, 253, 317, 6, 36 536/27; 260/112R; 424/92																															
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>14</sup> <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; border-bottom: 1px solid black;">Category <sup>6</sup></th> <th style="border-bottom: 1px solid black;">Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup></th> <th style="border-bottom: 1px solid black;">Relevant to Claim No. <sup>18</sup></th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td>US, A, 4,358,535, published 9 November 1982 Falkow et al.</td> <td style="text-align: center; vertical-align: top;">1-52</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y,E</td> <td>US, A, 4,466,917, published 21 August 1984 Nussenzweig et al.</td> <td style="text-align: center; vertical-align: top;">1-52</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td>N, Nucleic Acids Research Vol. 9, No. 13, Pages 2989-2998 (1981)</td> <td style="text-align: center; vertical-align: top;">1-52</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td>N, Davis et al., Microbiology Including Immunology and Molecular Genetics, Pages 610-619 (1980)</td> <td style="text-align: center; vertical-align: top;">1-31, 34-52</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">X</td> <td></td> <td style="text-align: center; vertical-align: top;">32 &amp; 33</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td>N, Joklik et al., Zinsser Microbiology 17th Edition, Pages 558-559 (1980)</td> <td style="text-align: center; vertical-align: top;">1-31, 34-52</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">X</td> <td></td> <td style="text-align: center; vertical-align: top;">32 &amp; 33</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td>N, Braude et al., Microbiology, Page 284 (1982)</td> <td style="text-align: center; vertical-align: top;">1-31, 34-52</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">X</td> <td></td> <td style="text-align: center; vertical-align: top;">32 &amp; 33</td> </tr> </tbody> </table>			Category <sup>6</sup>	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>	Y	US, A, 4,358,535, published 9 November 1982 Falkow et al.	1-52	Y,E	US, A, 4,466,917, published 21 August 1984 Nussenzweig et al.	1-52	Y	N, Nucleic Acids Research Vol. 9, No. 13, Pages 2989-2998 (1981)	1-52	Y	N, Davis et al., Microbiology Including Immunology and Molecular Genetics, Pages 610-619 (1980)	1-31, 34-52	X		32 & 33	Y	N, Joklik et al., Zinsser Microbiology 17th Edition, Pages 558-559 (1980)	1-31, 34-52	X		32 & 33	Y	N, Braude et al., Microbiology, Page 284 (1982)	1-31, 34-52	X		32 & 33
Category <sup>6</sup>	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>																														
Y	US, A, 4,358,535, published 9 November 1982 Falkow et al.	1-52																														
Y,E	US, A, 4,466,917, published 21 August 1984 Nussenzweig et al.	1-52																														
Y	N, Nucleic Acids Research Vol. 9, No. 13, Pages 2989-2998 (1981)	1-52																														
Y	N, Davis et al., Microbiology Including Immunology and Molecular Genetics, Pages 610-619 (1980)	1-31, 34-52																														
X		32 & 33																														
Y	N, Joklik et al., Zinsser Microbiology 17th Edition, Pages 558-559 (1980)	1-31, 34-52																														
X		32 & 33																														
Y	N, Braude et al., Microbiology, Page 284 (1982)	1-31, 34-52																														
X		32 & 33																														
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>*</sup> Special categories of cited documents: <sup>15</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>																																
<b>IV. CERTIFICATION</b> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">           Date of the Actual Completion of the International Search <sup>2</sup>            31 October 1984         </td> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">           Date of Mailing of this International Search Report <sup>2</sup>            01 NOV 1984         </td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;">           International Searching Authority <sup>1</sup>            ISA/US         </td> <td style="border-bottom: 1px solid black; padding: 5px;">           Signature of Authorized Officer <sup>10</sup>             Alvin E. Tanenholtz         </td> </tr> </table>			Date of the Actual Completion of the International Search <sup>2</sup> 31 October 1984	Date of Mailing of this International Search Report <sup>2</sup> 01 NOV 1984	International Searching Authority <sup>1</sup> ISA/US	Signature of Authorized Officer <sup>10</sup> Alvin E. Tanenholtz																										
Date of the Actual Completion of the International Search <sup>2</sup> 31 October 1984	Date of Mailing of this International Search Report <sup>2</sup> 01 NOV 1984																															
International Searching Authority <sup>1</sup> ISA/US	Signature of Authorized Officer <sup>10</sup> Alvin E. Tanenholtz																															

